



numbers were used for identification purposes during the conduct of the research program.

The experimental cigarettes, upon receipt, were placed in a rented commercial deep freeze storage facility at -5°F . The storage bin was locked and accessible only to authorized personnel. Cartons containing 10 packs each of the cigarettes were transferred as needed from the off-site storage location to the laboratory facility for preparation of the whole smoke condensates. Weekly supplies of cigarettes were stored at the laboratory in a locked file cabinet where they were allowed to reach ambient temperature. Subsequently, they were removed from the cartons and packages and stacked loosely in a Hot Pack temperature-humidity chamber at 60% relative humidity and 75°F . The cigarettes were held at these conditions for 24 hours prior to smoking.

Two Borgwaldt (RM20/68) smoking machines, supplied by the sponsor, were used to produce the whole smoke condensate used in the animal study. These machines smoked 20 cigarettes in 15 minutes under standard conditions as described by Ogg (1). The cigarette smoke was condensed in an Elmenhorst (2) cold trap immersed in an ethanol-dry ice bath. The condensate was produced in batch lots from smoking approximately 3000 cigarettes, and was maintained in a frozen state prior to chemical analysis and dilution for application to the mice.

A five-port analytical smoking machine was used to perform periodic quality control determinations on samples of the two lots of experimental cigarettes. Particulate matter produced by smoking the cigarettes was trapped on a Cambridge filter as described by Ogg (1). Chemical analyses were performed immediately.

Smoke condensate derived from 3000 cigarettes was processed in batches to obtain a known mixture of "tar", acetone, and water. The original condensate, after thawing, was quantitatively transferred under vacuum through a coarse porosity sintered

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CIGARETTE SMOKE CONDENSATE PREPARATION
AND DERMAL APPLICATION TO MICE

FINAL REPORT

Submitted to

American Tobacco Company
Brown & Williamson Tobacco Corporation.
Larus and Brother Company, Inc.
Liggett & Myers Tobacco Company
P. Lorillard Company
Philip Morris, Inc.
R. J. Reynolds Tobacco Company
Stephano Brothers
United States Tobacco Company
Covington and Burling

February 14, 1973



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a subsidiary of Environmental Science Corporation

SPONSOR: See Below

DATE: February 14, 1973

MATERIAL: Tobacco Smoke Condensates

LOT NO: 27, LH13515
64, LH13516

SUBJECT: FINAL REPORT
Cigarette Smoke Condensate Preparation
and Dermal Application to Mice
Projects No. 454-102 and 454-103

SPONSORS

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OBJECTIVE

The objective of this research was to evaluate the High Tor Foundation's claim that the skin tumor incidence is significantly lower in mice treated with whole smoke condensate from cigarettes containing Chemosol, as compared to the tumor incidence in mice treated with condensate from cigarettes without Chemosol.

The project was initiated in December 1969 and completed in November 1972.

MATERIALS AND METHODS

A. Smoke Condensate Preparation

The experimental cigarettes, fabricated and packed by the sponsor, were received at the laboratory in two lots numerically coded as No. 27 and No. 64. These lots were cross coded using the internal coding system of Hazleton Laboratories. Lot No. 27 was identified as LH13515, and No. 64 as LH13516. The assigned Hazleton code

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glass funnel into a tared 2000-ml round bottom flask using acetone as a vehicle. The acetone was removed on a flash evaporator at 35°C. The flask and contents were weighed to obtain an approximate weight of the "tar". Acetone and water were then added to obtain a final mixture approximating 50% "tar", 42% acetone, and 8% water. The actual composition of the mixture was determined with a gas chromatograph.

The gas chromatographic method of Schultz and Spears (3) was readily applicable to the determination of the water and acetone content of the mixture. A mixed analytical standard, containing 8 mg of water, and 42 mg of acetone in 4.0 ml of dioxane, was prepared for each batch adjustment. Acetone and water standard curves were obtained by peak height quantitation for 5 μ l, 10 μ l, and 15 μ l aliquots of the mixed standard. A 100 mg aliquot of the "tar" mixture was dissolved in 4 ml of anhydrous dioxane. A 10- μ l aliquot of the dissolved "tar" mixture was analyzed by gas chromatography using a thermal conductivity detector. The relative weights or percent composition of the two volatile fractions (acetone and water) of the mixture were computed from their respective standard curves. The final desired percent composition of the mixture was obtained by addition of acetone and/or water. The percent "tar" composition was computed by the difference between the sum of the acetone and water percent weight and the total (100%) weight of the aliquot analyzed.

A 10 ml aliquot of the adjusted mixture was retained for additional analyses. The remainder of each batch was utilized in the animal study. All fractions of each batch were stored at 0°C prior to use.

The pH was determined on the filtrate after mixing 0.5 ml of the "tar" mixture with 49.5 ml of water. The solution was filtered with Whatman No. 1 paper prior to the pH determination on a Beckman meter.

Nicotine Determinations

Approximately 200 mg of the smoke condensate were weighed into a tared No. 0

50154 7437



gelatin capsule. This was accomplished by taring the capsule in a Swagelok hexnut. After obtaining the weight of the capsule and hexnut, the condensate was quickly pipetted into the capsule. Twelve to 13 drops of the condensate mixture from a short disposable pipette yielded approximately 200 mg. The capsule was sealed and weighed again. The sample weight was determined by difference. The hexnut with the capsule was stored in a freezer prior to the clean-up procedure.

The clean-up and analytical methodology for nicotine utilized in this study was the Philip Morris method, described in detail in the appendix to this report.

Benzo[a]pyrene Determinations

Approximately 1 gram of the smoke condensate was weighed into a small tared beaker as quickly as possible to prevent weight loss due to evaporation of acetone. The condensate was quantitatively transferred to a large Cambridge filter pad (preconditioned for three hours in a furnace at 450°F) with 1 ml rinses of acetone. Transfers were continued until the rinses were free of fluorescence. The acetone was allowed to evaporate from the pad in a hood at room temperature. The pad was sectioned into small pieces and placed in a Lourde Multi-Mix homogenizer with 50 ml of hexane.

The method of Oakley, Johnson, and Stahr (4) was followed for the remainder of the analysis with the following stipulations: paper chromatography was used as the final separation step; a new stock standard solution was made every five to six weeks; fresh working standards were made for each run; and the paper chromatography developing solution was changed approximately every six weeks.

Condensate produced on the five-port analytical machine was analyzed periodically for quality control purposes. Total particulate matter was determined by the method of Ogg (1). Water was determined by the method of Schultz and Spears (3).

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Benzo[a]pyrene was determined by the method of Oakley, Johnson, and Stahr (4), and nicotine was determined by the Philip Morris method (see Appendix).

A gravimetric moisture determination was performed one time during the study to check the amount of moisture maintained in the Hot Pack humidity chamber. The tobacco from four cigarettes from each lot number was weighed immediately after removal from the humidity chamber. The tobacco was dried to constant weight in a vacuum oven at 60°C and weighed again. The weight loss was computed as moisture content on a percent basis.

B. Chronic Dermal Study - Mice

Initially, a preliminary toxicity study of three weeks duration was performed to determine the dose of the smoke condensates to be applied in the chronic study. In this short-term toxicity study 90 young adult ICR Swiss mice (Charles River Breeding Farms), 45 males and 45 females, were assigned to the following groups:

Group No.	Treatment	No. of Animals	
		Male	Female
1	0.1 ml acetone	15	15
2	Condensate 27, LH13515	15	15
3	Condensate 64, LH13516	15	15

Dermal applications were made three times weekly (Monday, Wednesday, and Friday) at a volume of 0.1 ml. The experimental smoke condensates were applied at levels from 40 mg/dose to 100 mg/dose, calculated on dry weight basis.

After completion of the above preliminary study the chronic repeated dermal study was initiated.

A total of 800 weanling ICR Swiss mice, 400 males and 400 females, were randomly assigned to the following five groups:

50154 7439



Group No.	Treatment	No. of Animals	
		Males	Females
1	Negative Controls - no treatment	50	50
2	Vehicle Controls - 0.1 ml acetone	100	100
3	Positive Controls - benzo[a]pyrene	50	50
4	Experimental Condensate - No. 27, LH13515	100	100
5	Experimental Condensate - No. 64, LH13516	100	100

The hair on the dorsal area of each animal was clipped at the beginning of the study, and this was repeated as necessary. This area, approximately 2 cm x 3 cm, was treated three times weekly with 0.1 ml acetone (vehicle) or the positive control chemical or experimental smoke condensate dissolved in this volume of solvent.

All animals were individually housed in wire mesh cages supported above the droppings. Water and appropriate food were freely available. Individual body weights were recorded initially, and at monthly intervals. Observations were made daily for mortality and twice weekly for evidence of systemic effect and skin lesions in the area of treatment. The study continued for 740 days.

Detailed records were maintained on the incidence, gross description, size, date of appearance, and disposition of tumors in the area of dermal applications during the progress of the study. Moribund animals were sacrificed, and necropsies performed. The following tissues were fixed in 10% neutral formalin:

brain	liver	small intestine	uterus
pituitary	spleen	large intestine	skin
thyroid	kidney	axillary lymph node	all abnormal organs
thymus	adrenal	urinary bladder	and tissues
lung	stomach	testis or ovary	

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Microscopic examinations were made on 20 skin tissues from the negative controls, 20 tissues from the vehicle control group, 10 from the positive control group, 105 tissues from mice treated with condensate No. 27, and 106 tissues from the mice treated with condensate No. 64.



The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

RESULTS

A. Chemical Analyses

Chemical analysis data obtained on the smoke condensate produced on the Borgwaldt RM20/68 machines are presented as process control determinations. Similar data obtained on the total particulate matter (TPM) produced by the five-port analytical smoking machine are presented as quality control determinations.

Tables 1 through 3 present process control data. Table 1 lists the total number of batches of condensate produced and the weight of dry "tar" obtained in each batch. A total of 30 batches of condensate were made of each experimental cigarette. The average dry "tar" determination for cigarette No. 27 was 57.2 grams for 22 batches. The average for cigarette No. 64 of 24 batches was 55.0 grams. Considering the variation in dry "tar" obtained from batch to batch these two average determinations are comparable.

Table 2 presents the percent composition of "tar", acetone and water and pH on each batch of condensate produced.

Table 3 presents the analytical data for nicotine and benzo[a]pyrene content of each batch of condensate. Nicotine is given in mg per gram of "tar" and benzo[a]pyrene as nanogram per mg of "tar". Considering the variation in these determinations from one batch to another the nicotine and benzo[a]pyrene contents of the smoke condensate of cigarette 27 and 64 were comparable.

Table 4 contains information, similar to that in Tables 1 through 3, obtained on total particulate matter produced on the five-port analytical smoking machine.

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These results are presented on a per cigarette basis. Cigarette No. 27 had an average "tar" determination of 28 mg/cigarette, 1.506 mg nicotine/cigarette, and 18.7 ng benzo[a]pyrene. Comparable determination of 27.3 mg "tar", 1.405 mg nicotine, and 19.3 ng benzo[a]pyrene were observed for cigarette No. 64. The results of the gravimetric moisture determinations are given in Table 5.

Figure 1 presents typical chromatograms obtained from the adjustment of a smoke condensate to a known percent composition. Figures 2 and 3 depict standard curves for water and acetone, respectively. A typical spectrum for a nicotine determination is presented in Figure 4. Figures 5 and 6 are photographs of the smoking instruments used in this study while Figure 7 contains a graphic representation of an Elmenhorst trap.

B. Chronic Dermal Study - Mice

In the preliminary toxicity study significant skin irritation was observed at the 50 mg/dose and higher levels of application of the whole smoke condensates. Therefore, the chronic dermal study was initiated at 25 mg/dose, three times weekly. After five weeks the dose was increased to 30 mg. After four weeks skin irritation was observed in a significant number of mice. The dose was reduced to 25 mg of whole smoke condensate from cigarette No. 27 and No. 64 and remained at this level throughout the study without significant signs of skin irritation.

Group 1 - Negative Controls

- None of the animals in this negative control group developed tumors in the dorsal skin area. This absence of lesions on the dorsal skin in the treatment area is normal in our experience with the ICR strain of Swiss mice in long-term studies such as this. Our observed incidence has been approximately one spontaneous neoplasm in the dorsal skin area in 300 control mice.

50154 7442



At termination of the study, 16 mice were surviving in this group and the average number of experimental days for the group was 556. Table 6 gives the number of experimental days for each animal in this group.

Microscopic examinations were performed on the skin tissues from 10 males and 10 females of this group. These data are summarized in Table 12. Thirteen of these tissues were normal. Mites were observed in four sections, dermatitis in four, and acanthosis in two skin sections. Two spontaneous tumors, outside the area of treatment, were observed. Animal No. 25 had a sebaceous gland adenoma and animal No. 52 had a papillary duct carcinoma of the mammary gland.

Group 2 - Vehicle Controls

None of the animals in this vehicle control group developed tumors in the dorsal skin area where the acetone applications were made. This observation is normal since acetone has never been reported to induce skin lesions in similar chronic studies. These mice received 30.5 ml acetone in 305 dermal applications.

At termination of the study, 33 mice were surviving in this group, and the average number of experimental days for the group was 584. Table 7 gives the number of experimental days for each animal in this group.

Microscopic examinations were performed on the skin tissues from 10 males and 10 females of this group. These data are summarized in Table 12. Twelve of these tissues were normal. Mites were observed in four sections, dermatitis in two, acanthosis in one, and hyperkeratosis in three skin sections. Two spontaneous tumors, outside the area of treatment, were observed. Animal No. 103 had a sebaceous gland adenoma and animal No. 220 had an adenosarcoma of the ceruminous gland.

Group 3 - Positive Controls

The 100 mice, 50 males and 50 females, in this group received 20 y

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benzo[a]pyrene in 0.1 ml acetone three times weekly. The maximum total dose was 2.5 mg in 126 dermal applications.

As shown in Table 8, 97 of the 100 mice developed gross lesions in the skin in the area of dermal applications of benzo[a]pyrene.

The median latent period for the 97 gross lesions was 162 days and the mean was 165 days.

The potency of the carcinogenic effect of repeated dermal applications of benzo[a]pyrene can be calculated by the method of Iball (5). The formula is given below:

$$\text{Carcinogenic Index (C.I.)} = \frac{X}{NL} \times 100$$

X = Number of animals bearing tumors

N = Number of animals alive at time of appearance
of first tumor

L = Mean latent period in days

In this group of mice, X = 97, N = 99, and L = 165 days. Thus, the C.I. was calculated:

$$\text{C.I.} = \frac{97}{99 \times 165} \times 100 = 0.59$$

Microscopic examinations were performed on the skin tissues from five males and five females of this group. As shown in Table 12, all ten of these lesions were squamous cell carcinomas.

These observed effects of benzo[a]pyrene are consistent with our previous studies and the published literature on this carcinogenic chemical. It is concluded that these experimental data are satisfactory as positive control information for this study.

Group 4 - Experimental Smoke Condensate 27 (LH13515)

The 200 mice, 100 males and 100 females, in this group received 25 mg of

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smoke condensate No. 27 in 0.1 ml of acetone three times weekly. The maximum total dose was approximately 7.7 grams in 305 dermal applications.

As shown in Table 9, 105 of the 200 mice developed gross lesions in the skin in the area of dermal applications of experimental smoke condensate No. 27.

The median latent period for the 105 gross lesions observed was 449 days and the mean was 438 days. A total of 168 tumors were identified in these mice - thus, the average number of tumors/tumor bearing animal was 1.6.

The carcinogenic index for this group of mice was calculated as follows:

$$\begin{aligned} X &= 105 \\ N &= 179 \\ L &= 438 \text{ days} \end{aligned}$$

$$\text{C.I.} = \frac{105}{179 \times 438} \times 100 = 0.13$$

Microscopic examinations were performed on the skin tissues from the 105 animals which had developed a tumor in the skin area of treatment during the study. These data are summarized in Table No. 12. A total of 80 of these lesions, present at necropsy and examined were neoplastic. There were 54 squamous cell carcinomas, 23 squamous cell papillomas, 2 sebaceous gland adenomas, and one mast cell sarcoma.

Group 5 - Experimental Smoke Condensate 64, LH13516

The 200 mice, 100 males and 100 females, in this group received 25 mg of smoke condensate No. 64 in 0.1 ml acetone three times weekly. The maximum total dose was approximately 7.7 grams in 305 dermal applications.

As shown in Table No. 10, 106 of the 200 mice developed gross lesions in the skin in the area of dermal applications of the experimental smoke condensate. The median latent period for the 106 gross lesions observed was 351 days and the mean was 372 days. A total of 165 tumors were identified in these mice - thus, the

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average number of tumors/tumor bearing animal was 1.5.

The carcinogenic index for this group of mice was calculated as follows:

X = 106
N = 186
L = 372 days

$$C.I. = \frac{106}{186 \times 372} \times 100 = 0.15$$

Microscopic examinations were performed on the skin tissues from the 106 animals which had developed a tumor in the skin area of treatment during the study. These data are summarized in Table 12. A total of 88 of these lesions, present at necropsy and examined were neoplastic. There were 58 squamous cell carcinomas, 18 squamous cell papillomas, one sebaceous gland adenoma, ten fibrosarcomas and one hemangiosarcoma.

DISCUSSIONS AND CONCLUSIONS

The significant parameters determined in the chronic mouse dermal study on the carcinogenic effects of smoke condensate 27 and 64 are given in Table 13 for comparison. The tumor incidence of 105 in 200 mice observed for condensate 27 and 106 in 200 mice for condensate 64 are comparable. The number of neoplastic lesions of the skin present at necropsy and histologically diagnosed are also comparable - 80 for condensate 27 and 88 for condensate 64. The cell types of neoplasia observed in the two groups are also comparable except for the absence of fibrosarcomas induced by condensate 27 and ten being observed for condensate 64.

The total number of gross skin tumors observed in the 105 animals treated with condensate 27 was 168 and for condensate 64, 165. Thus, the average number of tumors/tumor-bearing mouse was 1.6 and 1.5 respectively.

As shown in Table 13, the mean and median latent periods observed for

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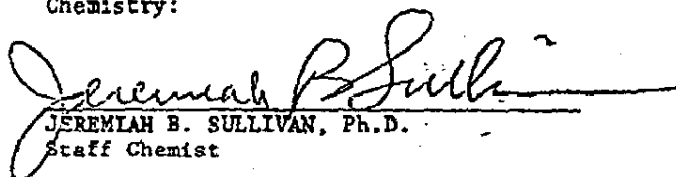
the two experimental groups of mice were significantly different. The mean latent period was 372 days for condensate 64, 66 days shorter than that of 438 days observed for condensate 27. The median latent period for condensate 64 was 351 days, 98 days shorter than the 449 days observed for condensate 27. This difference in the two latent periods is also reflected in the calculated carcinogenic index of 0.13 for condensate 27, and a slightly higher index of 0.15 calculated for condensate No. 64. Each of these values are much lower than the 0.59 observed for benzo[a]pyrene.

Based on the chemical and biological data observed in this study, we conclude that smoke condensates No. 27 and No. 64 are comparable.

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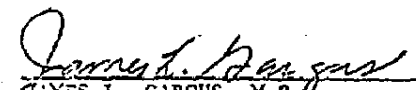
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